

# **OLIGONUCLEOTIDE FOR GENOTYPING *MYCOPLASMA* AND ITS RELATED STRAINS, MICROARRAY COMPRISING THE OLIGONUCLEOTIDE, AND METHOD FOR DETECTING STRAINS USING THE MICROARRAY**

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## **Technical Field**

The present invention relates to a method for detecting *Mycoplasma* and its related strains which are a source of contamination of cell lines and biological products and human pathogens. More particularly, the present  
10 invention relates to genus-specific and species-specific oligonucleotides for genotyping *Mycoplasma*, *Acholeplasm* and *Ureaplasma* strains, a microarray comprising the oligonucleotides, and a method for detecting strains using the microarray.

## 15 **Background Art**

*Mycoplasma* is a prokaryote pertaining to Mollicute family without cell wall, which was known as a hospital acquired pathogen causing pneumonia via infection of genital and respiratory organs of human as well as livestock such as pig and cow. Recently, *Mycoplasma* is more seriously  
20 understood as a major contaminant of cell culture and cell line.

Especially, as the development and production of biological products for protecting and treating human diseases increases, the contamination of various pathogens provided by microorganism or clinical sample in the process of production became a serious problem. Examples of the  
25 biological products are an oncolytic virus, vaccine, a gene therapy vector and a recombinant protein. They have been found to be contaminated by bacteria, fungus, virus, *Mycoplasma* and its related strains (Doblhoff-Dier *et al.*, 2001). The reason of the contamination is an organism contaminated in media components or experimental instruments and  
30 cross-contamination of microorganism and virus in air (Jung *et al.*, 2003). Also, the contamination can be occurred by a cross-contamination of

already-infected WCB (Working Cell Bank) which is used for mass production of biological products (Wisher *et al.*, 2002).

It is reported that, among these contamination sources, about 15~35% of cell culture or cell line is infected by *Mycoplasma* and its related strains (Hopert *et al.*, 1993). This also makes experimental results incredible because it can change characteristics of cells such as abnormal synthesis of DNA, RNA and protein by binding to host cell wall (Kong *et al.*, 2001). As gene therapy and cell therapy are getting into the spotlight recently, an assay for infection of stem cell and cord blood by *Mycoplasma* and its related strain became more important. Therefore, for the credible and reproducible experimental results and the quality control of commercialized biological products, it is essential to detect an infection with *Mycoplasma* and its related strains.

Under this situation, Europe community make it a rule that, for credibility of safety and quality of food and drug, GMP (Good Manufacturing Practice) and QC (Quality Control) should be submitted and cell banks such as MCB (Master Cell Bank) and WCB should be subjected to an assay for detection of virus, fungus and bacteria such as *Mycoplasma* (Dobhoff-Dier *et al.*, 2001).

About 100 kinds of bacteria pertaining to Mollicute family without cell wall have been found so far, including *Acholeplasma*, *Enteroplasma*, *Mesoplasma*, *Mycoplasma*, *Ureaplasma* and *Spiroplasma*. Among them, about 20 kinds of *Mycoplasma*, *Acholeplasma* and *Ureaplasma* are major contamination source of cell culture. These are referred to as “*Mycoplasma* and its related strains” in this specification. About 95% of the contaminants are covered by *M. arginini*, *M. fermentans*, *M. orale*, *M. hyorhinis*, *M. hominis*, *M. salivarium*, *M. pirum*, *A. laidlawii* (Dorigo-zetsma *et al.*, 1997). However, *Mycoplasma* is difficult to be cultured in extracellular media and turbidity is rare in the culture. Therefore, there has been a need to the rapid and accurate genotypic detection method which can trace a contamination source of *Mycoplasma* and its related strains.

Conventional *Mycoplasma* detection methods are the culturing method, the DNA fluorochoime stain method, the immunofluorescence method, and the polymerase chain reaction (PCR) method (Dorigo-zetsma *et al.*, 1997). However, the culturing method has a drawback that extracellular culturing is difficult, preparing its media is complex by adding supplements such as serum and culturing time is too long, about 4 days ~ 3 weeks according to the kinds of strains (Jensen *et al.*, 2003). The DNA fluorochoime stain method such as Hoechest 33258 stain has a drawback that culturing condition is too difficult to match and subjective inspectors can make a misjudgment (Chen *et al.*, 1997). The immunofluorescence method such as ELISA has a drawback that bacteria having similar antigen with *Mycoplasma* such as *Streptococcus milleri group* and *Staphylococcus aureus* may raise a false positive signal due to of low specificity (Hopert *et al.*, 1993). The PCR method makes use of 16S/23S intergenic spacer region (ITS) and a gene coding 169 kDa of P1 cyadhesion proteine which represent variety of *Mycoplasma* (Uphoff *et al.*, 2002). The P1 gene, a surface antigen gene, has several subtypes representing diversity and has been used as a target gene for serological detection using immune reaction and genotypic detection using restriction fragment length polymorphism (RFLP) to identify *Mycoplasma* (Campo *et al.*, 1998). However, most of conventional PCR methods use a primer designed based on 16S rRNA which is a common sequence of prokaryotes, and second PCR or nested PCR having high sensitivity can make a cross-contamination of *Mycoplasma* dispersed in air and an amplification of a bacteria similar with *Mycoplasma* in classification (Uphoff *et al.*, 2002).

To overcome the above limitations of the conventional detection methods, a genotypic detection method using probes have been developed recently, which make it possible to analyze many kinds of genes in a short time using DNA hybridization principle based on gene sequencing and detect specifically a single base change using a proper hybridization condition between specific probe and target DNA.

The present inventors developed ITS-derived oligonucleotides capable of detecting *Mycoplasma* and its related strains, which are important in genotypic detection, and a microarray comprising the oligonucleotides as a probe for detecting *Mycoplasma* and its related strains.

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# **Disclosure of the Invention**

It is a first object of the present invention to provide oligonucleotides for detecting *Mycoplasma* and its related strains designed based on their ITS base sequences.

10 It is another object of the present invention to provide novel ITS sequences of *Mycoplasma bovis*, *Mycoplasma cloacale*, *Mycoplasma falconis*, *Mycoplasma faucium*, *Mycoplasma spermatophilum* and *Mycoplasma synoviae*, which is useful for detecting *Mycoplasma* and its related strains.

15 It is another object of the present invention to provide a microarray comprising genus-specific and species-specific oligonucleotides for detecting *Mycoplasma* and its related strains as probes.

It is another object of the present invention to provide a method for detecting *Mycoplasma* and its related strains using the microarray.

20 It is another object of the present invention to provide a kit for diagnosing *Mycoplasma* and its related species infection individually or simultaneously, comprising genus-specific and species-specific oligonucleotides for genotyping *Acholeplasma* and related strains.

25 According to an aspect of the present invention, there is provided a purified ITS (internal transcribed spacer) target DNA for genotyping *Mycoplasma* strains, comprising any one sequence selected from SEQ ID Nos. 1 to 6.

30 SEQ ID Nos. 1 to 6 are base sequences of ITS (internal transcribed spacer) of *Mycoplasma bovis*, *Mycoplasma cloacale*, *Mycoplasma falconis*, *Mycoplasma faucium*, *Mycoplasma spermatophilum* and *Mycoplasma*

*synoviae*, which was newly obtained by base sequencing analysis.

The ITS target DNA of the present invention can be used indirectly for designing probes or primers used for genotyping *Mycoplasma* strains or directly for genotyping *Mycoplasma* strains via PCR amplification.

5 According to another aspect of the present invention, there is provided an oligonucleotide for genus-specific genotyping of *Mycoplasma* and *Ureaplasma* strains, comprising any one sequence selected from SEQ ID Nos. 7 to 21 or its complementary sequence.

10 According to another aspect of the present invention, there is provided an oligonucleotide for genus-specific genotyping of *Acholeplasma* strains, comprising any one sequence selected from SEQ ID Nos. 22 to 27 or its complementary sequence.

15 According to another aspect of the present invention, there is provided an oligonucleotide for species-specific genotyping of *Mycoplasma* and *Ureaplasma* strains, comprising any one sequence selected from SEQ ID Nos. 28 to 127 or its complementary sequence.

20 According to another aspect of the present invention, there is provided an oligonucleotide for species-specific genotyping of *Acholeplasma* strains, comprising any one sequence selected from SEQ ID Nos. 128 to 133 or its complementary sequence.

The oligonucleotides according to the present invention are designed based on multiple sequence alignment of ITS (internal transcribed spacer) sequences, which are present between 16S rRNA and 23S rRNA of *Mycoplasma* and its related species. The oligonucleotides can be used as  
25 primers for PCR amplification in order to genotype *Mycoplasma* and its related species or as probes for hybridization reaction in order to genotype *Mycoplasma* and its related species.

30 According to another aspect of the present invention, there is provided a microarray comprising more than one oligonucleotides selected from genus-specific and species-specific oligonucleotides for genotyping *Mycoplasma*, *Acholeplasma* and *Ureaplasma* strains according to any one

from claims 2 to 5 as probes attached on a support.

In the microarray according to the present invention, the probes may be any materials having base sequence, preferably any one selected from a group consisting of DNA (Deoxyribose Nucleic acid), RNA (Ribose Nucleic Acid), and nucleic acid analogues such as PNA (Peptide Nucleic Acid),  
 5 LNA (Locked Nucleic Acid) and HNA (Hexitol Nucleic Acid).

In the microarray according to the present invention, the support may be any materials to which the probes can be attached, preferably any one selected from a group consisting of slide glass, plastic, membrane,  
 10 semiconductive chip, silicon and gel. The microarray according to the present invention can be manufactured using conventional method such as pin microarray, ink jet, photolithography or electric array method.

The microarray according to the present invention can be used for simultaneously genotyping various *Mycoplasma* and its related species  
 15 which are known as a major contaminant of biological drug and cell line as well as a human pathogen from one sample, as the microarray comprises genus-specific and species-specific oligonucleotides for genotyping *Mycoplasma* and its related species as a set attached a support.

According to another aspect of the present invention, there is provided  
 20 a method for detecting *Mycoplasma*, *Acholeplasma* and *Ureaplasma* strains, comprising the following steps:

- a) extracting nucleic acids from a sample;
- b) amplifying target DNA among the extracted nucleic acids;
- c) hybridizing the amplified target DNA with probes of the microarray  
 25 according to the above present invention; and
- d) detecting signals generated from the hybridization reaction.

In the detection method according to the present invention, the sample may be biological drug, cell line, or human tissues or serum. The purifying step can be performed using conventional DNA or RNA purification method  
 30 or kit. The signal detecting step can be performed using a conventional fluorescence scanner after binding conventional fluorescent dyes such as

Cy5 or Cy3.

According to another aspect of the present invention, there is provided a kit for diagnosing *Mycoplasma* and its related species infection, comprising more than one oligonucleotide selected from genus-specific  
 5 and species-specific oligonucleotides for genotyping *Acholeplasma*, *Mycoplasma* and *Ureaplasma* strains according to the above present invention.

In the kit according to the present invention, the oligonucleotides are used as probes for hybridizing with target sample and may be contained in  
 10 a proper vessel. The probes may be labeled with a radioactive or non-radioactive labeling agent, the latter comprises conventional biotin, Dig(digoxigenin), FRET(fluorescence resonance energy transfer) or fluorescent dye (Cy5 or Cy3). Further, the oligonucleotides can be used as primers for PCR amplification. In this case, the kit may contain DNA  
 15 polymerase, 4 dNTPs and PCR buffer for PCR reaction. In addition, the oligonucleotides can be attached to a microarray as probes. In this case, the kit may contain hybridization reaction buffer, PCR kit containing primers for amplifying a target gene, washing solution for the unhybridized DNA, dyes, washing solution for unbound dyes and manual sheet for the  
 20 microarray.

Hereafter, the present invention will be described in more detail.

The present invention provides a method for detecting or genotyping *Mycoplasma* and its related strains which is a major contamination source of cell lines and biological products and a human pathogen, comprising the  
 25 following steps:

- a) if necessary, extracting nucleic acids from a sample such as cell lines, biological products or human tissue or serum;
- b) if necessary, amplifying target DNA of *Acholeplasma*, *Mycoplasma* and *Ureaplasma* strains among the extracted nucleic acids using more  
 30 than one proper primers;
- c) hybridizing the amplified target DNA with probes having a sense or

antisense or complementary sequences of genus-specific and species-specific oligonucleotides of *Acholeplasma*, *Mycoplasma* and *Ureaplasma* strains disclosed in Tables 2 and 3; and

d) detecting signals generated from the hybridization reaction.

5 From the detected signals in the step d), the existence of *Mycoplasma* and its related strains in the sample can be predicted.

The present inventors carried out a sequence analysis of ITS regions of many *Acholeplasma*, *Mycoplasma* and *Ureaplasma* strains to obtain genus-specific and species-specific oligonucleotides for detecting  
10 *Mycoplasma* and its related stains which can be a basis of developing a specific and sensitive hybridization assay. Also, the present inventors newly analyzed ITS sequences of newly found 6 *Mycoplasma* strains, which makes it possible to design probes capable of detecting more various *Mycoplasma* and its related strains.

15 Table 1 discloses ITS sequences of newly analyzed 6 strains among target sequences for detecting *Mycoplasma* strains, which correspond to SEQ ID Nos. 1 to 6. In the present invention, the probes for detecting *Mycoplasma* strains were designed based on the multiple alignment of ITS sequences of *Mycoplasma*.

20 FIGS. 1 and 2 show multiple sequence alignments of ITS regions of *Mycoplasma*, *Acholeplasma* and *Ureaplasma* for selecting genus-specific and species-specific probes of *Mycoplasma* and its related strains. Genus-specific oligonucleotides of *Mycoplasma* and *Ureaplasma* were designed from conservative sequence region indicated by a box in FIGS. 1a to 1f.  
25 Species-specific oligonucleotides of *Mycoplasma* and *Ureaplasma* were designed from polymorphic sequence region outside the box in FIGS. 1a to 1f. Genus-specific oligonucleotides of *Acholeplasma* were designed from conservative sequence region indicated by a box in FIGS. 2a to 2c. Species-specific oligonucleotides of *Acholeplasma* were designed from  
30 polymorphic sequence region outside the box in FIGS. 2a to 2c.

In step b) of the present invention, the target DNA of *Acholeplasma*,



*Mycoplasma* and *Ureaplasma* strains were amplified using more than one pair of proper primers. FIG. 3 shows PCR amplification of ITS target sequences of *Mycoplasma* and its related strains using a primer pair, MP16SF-2 and MP23SR-2. In FIG. 3, 1 is a PCR product of *M. arginini*, 2 is a PCR product of *M. arthritidis*, 3 is a PCR product of *M. fermentans*, 4 is a PCR product of *M. hominis*, 5 is a PCR product of *M. hyorhinae*, 6 is a PCR product of *M. neurolyticum*, 7 is a PCR product of *M. opalescens*, 8 is a PCR product of *M. orale*, 9 is a PCR product of *M. pirum*, 10 is a PCR product of *M. penetrans*, 11 is a PCR product of *M. pulmonis*, 12 is a PCR product of *M. salivarium*, 13 is a PCR product of *M. cloacale*, 14 is a PCR product of *M. falconis*, 15 is a PCR product of *M. faucium*, 16 is a PCR product of *M. hyosynoviae*, 17 is a PCR product of *M. muris*, 18 is a PCR product of *M. primum*, 19 is a PCR product of *M. spermatophilum*, 20 is a PCR product of *M. synoviae*, 21 is a PCR product of *M. pneumoniae*, 22 is a PCR product of *M. genitalium*, 23 is a PCR product of *M. bovis*, 24 is a PCR product of *U. urealyticum*, 25 is a PCR product of *A. laidlawii*.

In step c) of the present invention, the amplified target DNA were hybridized with probes for detecting *Mycoplasma* and its related strains. Preferably, the probes may be a combination of more than one probes capable of simultaneously detecting many *Mycoplasma* and its related strains from a single sample. Practically, the probes are optimized to simultaneously hybridize with multiple target DNAs of *Mycoplasma* and its related strains under the same hybridization and washing conditions.

The present invention provides a microarray comprising a set of probes for detecting *Mycoplasma* and its related strains, which can simultaneously detect many *Mycoplasma* and its related strains from a single sample with a single experiment.

In the present invention, the term 'probe' means a single-stranded oligonucleotide having a sequence complementary to target DNA of *Mycoplasma*, *Acholeplasma* and *Ureaplasma*. The probe may have a sense, antisense or complementary sequence of SEQ ID Nos. disclosed in

5 this specification as long as it can hybridize with one of double strands of  
 target DNA. The oligonucleotide may be ribonucleotide (RNA),  
 deoxynucleotide (DNA), peptide nucleic acid (PNA) or locked nucleic acid  
 (LNA), and contain modified nucleotides such as Inosine only if it does not  
 10 change their hybridization characteristics. Preferably, the genus-specific  
 oligonucleotides for detecting *Mycoplasma*, *Acholeplasma* and  
*Ureaplasma* may have a base sequence of SEQ ID Nos. 7 to 27.  
 Preferably, the species-specific oligonucleotides for detecting *Mycoplasma*,  
*Acholeplasma* and *Ureaplasma* may have a base sequence of SEQ ID  
 10 Nos. 28 to 133.

FIG. 4 shows a microarray comprising probes for detecting genotypes  
 of *Mycoplasma* and its related strains as a set on a support. In FIG. 4,  
 each species name and SEQ IN Nos. are described which correspond to  
 individual probes. The terms 'MP-C' and 'AP-C' mean *Mycoplasma* and  
 15 *Ureaplasma* genus and *Acholeplasma* genus. FIG. 4 is no more than an  
 example of probe compartment of the present invention, so compartment  
 and layout of each probe can be varied.

In the present invention, newly analyzed ITS sequences of 6  
*Mycoplasma* strains as a target DNA for detecting *Mycoplasma* and its  
 20 related strains are as shown in Table 1. The genus-specific  
 oligonucleotides for detecting *Mycoplasma*, *Acholeplasma* and  
*Ureaplasma* used in the present invention are as shown in Table 2. The  
 species-specific oligonucleotides for detecting *Mycoplasma*, *Acholeplasma*  
 and *Ureaplasma* used in the present invention are as shown in Table 3.

25

【Table 1】

Species	Sequence (5' → 3')	SEQ ID NO.
<i>M.bovis</i>	TTCTACGGAGTACACTTGTCTTTTATCACTATAAAAAAAGACTTATAACCAAAAT TACTAGACCTATATTTATTTATAAACGTCATGGCTTTTATTAAATAGGTCAAAAGCTA TATATCTAGTTTGTAGAGAACATTCCTCATATGTTCTTGTGAAAACTGAAATAGTAAA ATATTTTTCGATATTTACAACGACATCAAAAAATCAAAATTAATGGTTAATTTGTCTTGTG ATTCAATCGAGTAAGTCAATTTAATAATGATTCATTGAAAATGCTTAAAAATACACATC TAAAC TAACAACAATAGGAAAA TACTACTTTTAAATAAGGAAGAGTTTGTGGTGG ATGC	1
<i>M.cloacae</i>	CTTCTACGGAGTACAATTCACATGTTATGGAAATTAATTTGTATCCAGTTTGTGAGA GAAC TTTCTCTCAATTTGTCTTTGTGAAAAC TGAAATATAGACATGTGAAATCAATAAA TTAATATTTCAAAATGTTTAGATCAACCTATAGAATATTC AAGACATATACAAAAATA GGTCATAC TTATTTTATAAAATAC T	2
<i>M.falconis</i>	CTTCTACGGAGTACAAC TCTGTTATGGAAATAATTTGTATCCAGTTTGTGAGAGT ACTAACTCTCTTTTGTCTTTGTGAAAAC TGAAATATCGACATTTGAAAAATTTAATTT AATATTTCAAAGTTTAGATCAACCTATAGAAATACAAAAATATAGACAACAAATAGGT CATACAACAAACA TAACAAAAACAAT	3
<i>M.faucium</i>	GAAATGGTGGCTTCGAGACTAAAAGTTATGTGAAAAACATCGTATCCAGTTTGTGAGA GAAC TAACTTCCTCTCTTTGTCTTTGTGAAAAC TGAAATATAGACATTTGAAAAATTA AAATTAATTTCAAAGTTTAGATCAACCTATAGAAATACAAAAATCAATACAATAGG TCAATAC TATACAA TTGCA TAACAAAAAAATAC TATTAACAAGATAAGAGTTTGTG GTGGAATGCAATTTGTA	4
<i>M.spermatophilum</i>	GTGGGGATGGATCACCCTCTCTACGAGGTACAACATACATTCAAAATTTTGACT GAAATGTTATTAACCTATATTTTTCAC TAGGCC TTTTAAATATATTTTGTATG TGA CTTTATGGCC TAAAAGCTTTATATCTAGTTTGTAGAGGACATCCCTCTAAATGTCTCT TTGAAAAC TGAATAGTAAATTTTGTGATATTTACAACGACATCTAAATAATTGAAAT AAGTCAATTTGTTTAGATTTCACTCGAGATAGTCAATTTAAAAAAATGATTCATTTGAA ATGTCTTAAAAATACACATCAAAACAAACAACTATACAAATAGGAAATTTATATACT	5
<i>M.synoviae</i>	TCC TTACGGAGTACATTAATTTTACAAAAGGCATTTTATTAAC TGAAGCTTTTAG AGAAAAATCTAAAAGCGGTTGTGTATCGCTTTTGTGCTTGGGCTATTTGTATTTA GTTTGTAGAGAACCAACCTCTCTTAAAAATGTCTTTGTGAAAAC TAAATAGTAATAAA GATATTACAACGACATCAAAAAATATAAATTAATTAAGGTTAAATTTGTCTTTGTATACCG AGTTTAAATTAATGTAAATAAATTTATTAATAATGCTTTGTGAAATACATCAATAACAATA TAACAAATAGGACATTTGTATCTAAC TTTTAAAAAAGT	6

5      【Table 2】

Genus	Probe	Sequence	SEQ ID NO
<i>Mycoplasma</i>	MP-CP1	TTCTTTGAAAAC TGA	7
	MP-CP2	RTWTC TTTVAAAAC TTTTATWN	8
<i>M. arginini</i> , <i>M. arthritidis</i> <i>M. cloacale</i> , <i>M. falconis</i> <i>M. faucium</i> , <i>M. hominis</i> <i>M. hyosynoviae</i> , <i>M. orale</i> <i>M. salivarium</i>	MP-CA1	MW TYG TR TCCAG TT TTGAGAG	9
	MP-CA2	TTTAG ATCAACCTA TAGAATA	10
	MP-CB1	RTATYTAG TT TTGAGAGHRCA	11
	MP-CB2	WW IHA TTYA TTTAAATG TCTT	12
<i>M. bovis</i> , <i>M. fermentans</i> <i>M. opalescens</i> , <i>M. primum</i> , <i>M. spermatophilum</i> , <i>M. synoviae</i>	MP-CB3	GG KYAATT TG TTTWGAT	13
	MP-CB4	RTATTTTACAMCGMCAYC	14
	MP-CC1	CC TCC TTTC TATCGGAGTAMA	15
	MP-CC2	CGGATTC TATTTAG TTTTGAG	16
<i>M. neurolyticum</i> , <i>M. pulmonis</i>	MP-CD1	TAAAATAGATACCTTAAKATA	17
	MP-CD2	GTATYYAG TTTTGAAAG	18
	MP-CD3	CTTGCCAATAGWTWT	19
<i>M. genitalium</i> , <i>M. pirum</i> <i>M. pneumoniae</i>	MP-CE1	AWACHACAACTCTTC TAGTTC	20
	MP-CE2	AATAAGTTAC TAAGGGCTTAT	21
<i>Acholeplasma</i>	AP-CP1	TCATCATATTCAGTTTG	22
	AP-CA1	GGGCC TR TAGC TCAGY TGG TT	23
	AP-CA2	AGAGCFCWCGCYTGA TAAGCG	24
	AP-CA3	WGRGG TCGATGGTTCRAG TCC	25
	AP-CB1	TCATCATATTCAGTTTIGARR	26
	AP-CB2	AGTC TTTGAAAAG TAGATAAA	27

5      【Table 3】

Species	Probe	Sequence	SEQ ID NO.
<i>M. arginini</i>	MP-arg1	AGATTATATCATACAATAGA	28
	MP-arg2	GAGTACATAAAATGTTATGGAA	29
<i>M. arthritidis-faucium</i>	MP-arf1	TGAAGCCCGATGGTGCTTCG	30
	MP-arf2	TGAGAGAACTAAACTTCTCTC	31
	MP-arf3	GAAACAAAAATCAATACAATA	32
<i>M. fermentans</i>	MP-fer1	ATGTACTATTAACCTATTTCAC	33
	MP-fer2	TACAAAAGAGTACTTTTAAA	34
	MP-fer3	TTTTATGGGCTAAAGCTTT	35
	MP-fer4	GAACAAATTTTTCCTCTCA	36
	MP-fer5	ATAACAAACTATAACAATAGG	37
<i>M. hominis</i>	MP-hom1	ATTTATCTCTCGGTTCTTT	38
	MP-hom2	ATATTTATATTTTATAAGACA	39
	MP-hom3	ATTGATATATTAATTAAATTT	40
<i>M. hyorhinis</i>	MP-hyo1	GAATAGCAAATAACAATATGATT	41
	MP-hyo2	CGGAGTACATTAGTCTTAATT	42
	MP-hyo3	TTACATAATCGATTCTGTCT	43
	MP-hyo4	AGCTTTAAGTCTCAATTATA	44
	MP-hyo5	TTCAATTTATTTATTTCAACG	45
	MP-hyo6	AACGATCTTTTATAAACCAG	46
	MP-hyo7	TTAAATTTCTAAAAATAGATTA	47
	MP-hyo8	AGATATTTATCTTTAGCAATA	48
<i>M. neurolyticum</i>	MP-neu1	GGTTATTATGGGCTTGCTA	49
	MP-neu2	GGTTATTTAAAAATCCTTTAA	50
	MP-neu3	TAAATTTTCTTCTTAATTAA	51
<i>M. opalescens</i>	MP-opa1	CATCATAATGTAACCAATAC	52
	MP-opa2	ACAAAAATCATTAATTTTAAAT	53
	MP-opa3	TTTAATGATTAATTAACCTTTT	54
	MP-opa4	TTATGTGCTTGTTTTATATGG	55
	MP-opa5	TATGGCTACAAAGCTTATAT	56
	MP-opa6	GATAAAAAACAAATCATAAATTT	57
<i>M. orale</i>	MP-ora1	CATAAATAGTTAATGGCTCA	58
	MP-ora2	ATAGAGACAAAACAAAAACA	59
	MP-ora3	GGTCAAAAATACTTATACGTA	60
<i>M. pirum</i>	MP-pir1	TAGTCTTTTG TGTAATAACA	61
	MP-pir2	CTTTATACACCTTATTACAAT	62
	MP-pir3	TAAAAATCCAATTTAAATGTTA	63
	MP-pir4	GCAAATTTGATGTCAACATTT	64
	MP-pir5	AATTAATCTCTCCATTACTT	65

	MP-pir6	TTAAAGTAGTAGAGATGGTTC	66
	MP-pir7	CAAAATCAAAATGCTAA TGG A	67
	MP-pir8	ATGC TAA TGG ATA TCAAAAAA	68
<i>M. penetrans</i>	MP-pen1	AAGAGTAAGTTC TAG GTCG	69
	MP-pen2	CATTAAAGCTAAG TAACAAAT	70
	MP-pen3	TCC TAAAC TGAAATTTATCT	71
	MP-pen4	TTA TATAAGAGTAAGTTC TAG	72
	MP-pen5	ATTTTCTC TCAAGATAGTTC	73
	MP-pen6	TC TAATCATACTTG TTATTTT	74
<i>M. pulmonis</i>	MP-pul1	AATTTTGA TCCGAGTCA TT	75
	MP-pul2	CATTTTCTA TCAATAGTTAT	76
	MP-pul3	TA TG TG TA TC TTGCCAATTAG	77
	MP-pul4	TTCTATCTTTCAAAACAAATA	78
	MP-pul5	TA TAAATTAATATGA TAACG T	79
	MP-pul6	TCATCAAAATG TAAAAATTTT	80
	MP-pul7	AAAAATAAAA TAG ATACC TTA	81
	MP-pul8	AAA TAAATTC AACAATAGGA	82
<i>M. salivarium</i>	MP-sal1	TAATGGATT TAAATTTTCG TG	83
	MP-sal2	ATCAAAATCAA TATAATATTT	84
<i>M. cloacae</i>	MP-clo1	AGTACAA TTC TAC TGTTATG	85
	MP-clo2	TAGAATA TTCAAGACATATAC	86
<i>M. falconis</i>	MP-fal1	GAGTACAAC TTCTGTTATG	87
	MP-fal2	AGAATACAAAAATATAGACAA	88
	MP-fal3	ATTGAAAAATTATTAATTAAT	89
<i>M. hyosynoviae</i>	MP-hyos1	CTAGACTAAAGTTAA TGGTAC	90
	MP-hyos2	AATTATCAAATTAATATTTCA	91
<i>M. muris</i>	MP-mur1	TATAGAAAACCCCCACATCA	92
	MP-mur2	TATTAGAATA TTTAAATATT	93
	MP-mur3	GATTA TTACACCATATTAGAA	94
	MP-mur4	TCAATAAACC TAAATAAAAAA	95
<i>M. primum</i>	MP-pri1	G TAG ACATAACCCAGC TA	96
	MP-pri2	CAAACGTCTA TCGC TTTTAG	97
	MP-pri3	TCATGGGCTTTAATAGGGTC	98
	MP-pri4	ACCCCAAC TCCCATCAAAAAAT	99
<i>M. spermatophilum</i>	MP-spe1	TTCATCGAGATAGTCATTTTA	100
	MP-spe2	CAAACATACATTCAAATTTT	101
	MP-spe3	TTTIGACTGAATG TTATTAAC	102
	MP-spe4	TTTGTTATG TGAC TTTTAGG	103
	MP-spe5	AAAACAAACAA TC TATACAA T	104

<i>M. synoviae</i>	MP-syn1	TTGGCTTGGGCTATGTATT	105
	MP-syn2	GCGGTIGTGTATCGCTTTT	106
	MP-syn3	ACCTCTCTTAAAAATTGTCTT	107
	MP-syn4	CCGAGTTTAAATTATGAATA	108
	MP-syn5	CATCATACAACATAACAATA	109
<i>M. pneumoniae</i>	MP-pne1	GTAAATTAAACCCAAATCCC	110
	MP-pne2	ATCTTTAATAAAGATAAAATAC	111
	MP-pne3	CTAAACAAAACATCAAAATCC	112
	MP-pne4	AAAGAACAATTCCTGCTTCTT	113
<i>M. genitalium</i>	MP-gen1	CACCCCTTAATTTTTCGG	114
	MP-gen2	AATGGAGTTTATTTTTATTTA	115
	MP-gen3	CCCAATCAAATGTTGGTCTC	116
	MP-gen4	CAACTAACACACTTGGTCAGT	117
	MP-gen5	AGAATGTTTGAACAGTTC	118
	MP-gen6	TAGTTCCAAAAATAAATACCA	119
<i>M. bovis</i>	MP-bov1	TATAACCAAAATTAAAGACCTA	120
	MP-bov2	GTCATGGCTTTATTAATAGG	121
<i>U. urealyticum</i>	UP-ure1	CATTAAGTTGTCAGTGAA	122
	UP-ure2	TAATTTACGTACTAATAAGTG	123
	UP-ure3	TTTATTAAAAATCCATATGAAT	124
	UP-ure4	AAGCCACTTTTAAAAATTT	125
	UP-ure5	CCATAATAATTAATTATTAT	126
	UP-ure6	ATTATCAACAAATCTTCTAA	127
<i>A. laidlawii</i>	AP-lai1	AACACTTAGCACAAAGATGAC	128
	AP-lai2	CTTCTAAGGAGAAAGGCTAA	129
	AP-lai3	ATGACTACTAGTAAGTAGTAA	130
	AP-lai4	GTAGTAATATTCCTAAATTT	131
	AP-lai5	TTAAAGTAATTAAGTGTTTC	132
	AP-lai6	TAAATGATGCTGAAAAGAAA	133

\* Mixed Base Code Name

M : A + C, W : A + T, Y : C + T, R : A + G

K : G + T, V : G + A + C, N : A + G + C + T

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### **Brief Description of the Drawings**

FIGS. 1a to 1f show multiple sequence alignments of each ITS region of *Mycoplasma* and *Ureaplasma* for selecting genus-specific probes.

10 FIGS. 2a to 2c show multiple sequence alignments of each ITS region of *Acholeplasma* for selecting genus-specific probes.

FIG. 3 shows a result of PCR amplification using primer pairs which can amplify ITS target sequences of many *Mycoplasma* and its related strains

15 FIG. 4 shows a microarray comprising probes for detecting genotypes of *Mycoplasma* and its related strains as a set on a support.

FIGS. 5a to 5k show results of image analysis of specific hybridization reaction of each probes for detecting genotypes of *Mycoplasma* and its

related strains and results of numerical analysis calculated from pixel intensity.

### **Best mode for carrying out the Invention**

5       The present invention will be described in greater detail by means of following examples. The following examples are for illustrative purpose and are not intended to limit the scope of the invention.

#### Example 1: Incubation of *Mycoplasma* and its related strains and 10 Isolation of Genomic DNA

Total 25 kinds of strains, including 1 kind of *Acholeplasma*, 23 kinds of *Mycoplasma*, and 1 kind of *Ureaplasma* were obtained from the American Type Culture Collection (ATCC). The strains were cultured in each culturing media under each culturing conditions according to manual  
15 provided by ATCC. From the cultured media, strain colonies were obtained with a white gold ear and input in 1.5ml tube, 100μl of InstaGene matrix (Bio-Rad, USA) was added thereto and suspended, and reaction was performed at 56°C for 30 minutes in constant temperature bath. And then, the reactant was shook for 10 seconds, heated at 100°C for 8 min,  
20 shook again for 10 sec, centrifuged at 12,000 rpm for 3 min, transferred to new tube, and freeze-stored at -20°C. The product was used as template DNA of PCR reaction.

The strains used were as followed:

*Acholeplasma laidlawii* (ATCC 25937)  
25 *Mycoplasma arginini* (ATCC 23838)  
*Mycoplasma arthritidis* (ATCC 19611)  
*Mycoplasma bovis* (ATCC 27368)  
*Mycoplasma cloacale* (ATCC 35276)  
*Mycoplasma falconis* (ATCC 51372)  
30 *Mycoplasma faucium* (ATCC 25293)  
*Mycoplasma fermentans* (ATCC 19989)



- Mycoplasma genitalium* (ATCC 33530)
- Mycoplasma hominis* (ATCC 23114)
- Mycoplasma hyorhinis* (ATCC 17981)
- Mycoplasma hyosynoviae* (ATCC 25591)
- 5 *Mycoplasma muris* (ATCC 33757)
- Mycoplasma neurolyticum* (ATCC 19988)
- Mycoplasma opalescens* (ATCC 27921)
- Mycoplasma orale* (ATCC 23714)
- Mycoplasma penetrans* (ATCC 55252)
- 10 *Mycoplasma pirum* (ATCC 25960)
- Mycoplasma pneumoniae* (ATCC 15531)
- Mycoplasma primum* (ATCC 15497)
- Mycoplasma pulmonis* (ATCC 14267)
- Mycoplasma salivarium* (ATCC 23064)
- 15 *Mycoplasma spermatophilum* (ATCC 49695)
- Mycoplasma synoviae* (ATCC 25204)
- Ureaplasma urealyticum* (ATCC 27618)

20 Example 2: Preparation of probes for detection of *Mycoplasma* and its related strains

The probes used for detection of *Mycoplasma* and its related strains were selected based on a result of multiple alignment of ITS sequences of *Mycoplasma*, *Acholeplasma* and *Ureaplasma*. Among *Mycoplasma* and its related species, 16S rRNA sequences has high similarity of 74~97%,  
 25 whereas ITS sequences has lower similarity of 25.4~78.8% except for between *M. salivarium* and *M. hyosynoviae*, and *M. hominis* and *M. falconis*. In other words, ITS contains a region more polymorphic than 16S rRNA which is useful for designing probes for detection of *Mycoplasma* and its related strains. However, to complement specificity between  
 30 *M. salivarium* and *M. hyosynoviae*, and *M. hominis* and *M. falconis* having a high ITS similarity, more restrictive and strict probes were designed.

In the present invention, the oligonucleotide probes for detection of *Mycoplasma* and its related strains were prepared by synthesizing 15-25 bases of specific probe with 15 bases of dT spacer at 5' end. Probes for detection of *Mycoplasma* and its related strains are not restricted to the sequences disclosed in Tables 2 and 3 and any primer and probes comprising the sequences can be used in the present invention.

1. Preparation of probes for detection of *Mycoplasma* and *Ureaplasma*

① Preparation of probes for genus-specific detection of *Mycoplasma* and *Ureaplasma*

For genus-specific hybridization with all *Mycoplasma* and *Ureaplasma* genus, probes of SEQ ID Nos. 7 and 8 in Table 2 were designed from conserved sequences of ITS of *Mycoplasma*. Further, each Group-based conserved sequences targeted to *Mycoplasma* ITS were designed as follows. For detecting Group I (*M. arginins*, *M. arthritidis*, *M. cloacale*, *M. falconis*, *M. faucium*, *M. hominis*, *M. hyosynoviae*, *M. orale*, *M. salivarium*), probes of SEQ ID Nos. 9 and 10 were designed. For detecting Group II (*M. bovis*, *M. fermentans*, *M. opalescens*, *M. primatum*, *M. spermatophilum*, *M. synoviae*), probes of SEQ ID Nos. 11, 12, 13 and 14 were designed. For detecting Group III (*M. muris*, *M. penetrans*, *U. urealyticum*), probes of SEQ ID Nos. 15 and 16 were designed. For detecting Group IV (*M. neurolyticum*, *M. pulmonis*), probes of SEQ ID Nos. 17, 18 and 19 were designed. For detecting Group V (*M. genitalium*, *M. pirum*, *M. pneumoniae*), probes of SEQ ID Nos. 20 and 21 were designed.

② Preparation of probes for species-specific detection of *Mycoplasma* and *Ureaplasma*

For species-specific hybridization with each *Mycoplasma* and *Ureaplasma* species, 100 kind of probes of SEQ ID Nos. 28 to 127 in Table 3 were designed from species-specific sequences of ITS of *Mycoplasma* and *Ureaplasma*, which can detect 25 kind of *Mycoplasma* strains.

## 2. Preparation of probes for detection *Acholeplasma*

### ① Preparation of genus-specific probes for detection *Acholeplasma*

For genus-specific hybridization with all *Acholeplasma* genus, probes  
 5 of SEQ ID No. 22 in Table 2 was designed from conserved sequences  
 targeted to both of ITS1 and ITS2 of *Acholeplasma*. Further, each Group-  
 based conserved sequences targeted to each *Acholeplasma* ITS1 and  
 ITS2 were designed as follows. For Group I targeted to ITS1, probes of  
 SEQ ID Nos. 23, 24 and 25 were designed. For Group II targeted to ITS2,  
 10 probes of SEQ ID Nos. 26 and 27 were designed.

### ② Preparation of species-specific probes for detection *Acholeplasma*

For species-specific hybridization with each *Acholeplasma* species,  
 probes of SEQ ID Nos. 128 to 133 in Table 3 were designed from species-  
 15 specific sequences of ITS of *Acholeplasma*.

## Example 3: Preparation of target DNA

1. Preparation of target DNA for detection of *Mycoplasma* and its  
 related strains

20 For preparing target DNA for detection of *Mycoplasma* and its related  
 strains, 187~290bp size of ITS regions were selectively amplified using  
 5'-biotin-GTG(C/G)GG(A/C)TGGATCACCTCCT-3' (MP16SF-2) and  
 5'-biotin-GCATCCACCA(A/T)A(A/T)AC(C/T)CTT-3' (MP23SR-2), and  
 5'-biotin-AAAGTGGGCAATACCCAACGC-3' (M78) and 5'-biotin-  
 25 CCACTGTGTGCCCTTTGTTTCCT-3' (R34) which were biotin-labeled  
 respectively (Tang *et al.*, 2000.). To prepare genomic DNAs of  
*Mycoplasma* and its related strains isolated in Example 1, PCR were  
 carried out using the above primers in the following conditions:  
 denaturation at 94°C for 3 minutes, 30 cycles of amplification at 94°C for 30  
 30 seconds, at 55°C for 2 minutes and at 72°C for 2 minutes, and final

extension at 72°C for 10 minutes. After the reaction, the reaction products were analyzed by ELECTROPHORESIS on a 2% agarose gel. FIG. 3 is an electrophoresis image taken after the PCR performed using primers capable to amplify ITS target sequences of several *Mycoplasma*.

5

#### Example 4: Probe immobilization on support

Among the probes prepared in Example 2, each representative probes for *Mycoplasma*, *Acholeplasma* and *Ureaplasma* were selected. Each of the selected probes was transferred to 384-well microplate, diluted to a concentration of 50 pmole by adding spotting solution, and immobilized on a slide glass using a microarrayer (Cartesian Technologies, USA). In FIG 4, each probes for detection of *Mycoplasma* and its related strains correspond to SEQ ID Nos. 7, 28, 30, 33, 38, 41, 49, 52, 58, 61, 69, 75, 83, 85, 87, 30, 90, 92, 96, 100, 105, 110, 114, 120, 122, 22, 128, and 7 in order. Two spots of each kind of the probes were attached to the support and left in a slide box at room temperature for 24 hours or in a dry oven at 50°C for about 5 hours to be fixed to the surface of the support.

#### Example 5: Unimmobilized probe washing

The slide glass after the process in Example 4 was washed with a 0.2% SDS buffer solution and then distilled water at room temperature to remove unimmobilized probes. The washed slide glass was immersed in a sodium borohydride (NaBH<sub>4</sub>) solution for 5 minutes and then washed again at 100°C. Final washing with a 0.2% SDS solution and then distilled water was followed by centrifugation to fully dry the slide glass.

#### Example 6: Hybridization

The biotin-labeled target products prepared in Example 3 were thermally treated to be denaturated into single strands and cooled to 4°C. A hybridization reaction solution containing 2μl of the target products was prepared. This hybridization reaction solution was portioned on the slide

glass after the process in Examples 4 and 5, and the slide glass was covered with a cover slip and reacted at 25°C for 1 hours.

Example 7: Unhybridized tart DNA washing

5 TO WASH OUT UNHYBRIDIZED TARGET DNAS, THE COVER SLIP WAS REMOVED USING A 2X SSC WASHING SOLUTION (300MM NACL, 30MM NA-CITRATE, PH 7.0), AND THE SLIDE WAS WASHED WITH 2X SSC AND THEN 0.2X SSC, FOLLOWED BY CENTRIFUGATION TO FULLY DRY THE SLIDE GLASS.

10

Example 8: Staining and Result analysis

To determine hybridization of PCR products and probes, Cy5-streptavidin or Cy3-streptavidin (Amersham pharmacia biotech, USA) was diluted with 6x SSC and BSA (Bovine Serum Albumin), about 40 $\mu$ l of  
 15 dilutes was portioned on slide glass, and the slide glass was covered with a cover slip to block light and reacted at 50°C for about 20 minutes. After the reaction, the cover slip was removed using a 2X SSC solution, and the slide was washed with 2X SSC and then 0.2X SSC. The hybridized result was scanned using a non-confocal laser scanner (GenePix 4000A, Axon  
 20 Instruments, U.S.A.) and analyzed by image analysis.

FIG. 5 shows results of image analysis of specific hybridization reaction of each probes for detecting genotypes of representative 11 kinds of *Mycoplasma* and its related strains and results of numerical analysis  
 25 calculated from pixel intensity.

FIG. 5a shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 85) of *M. cloacale*. FIG. 5b shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 87) of *M. falconis*.  
 30 FIG. 5c shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 90) of *M.*

*hyosynoviae*. FIG. 5d shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 49) of *M. neurolyticum*. FIG. 5e shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 52) of *M. opalescens*. FIG. 5f shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 69) of *M. penetrans*. FIG. 5g shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 61) of *M. pirum*. FIG. 5h shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 83) of *M. salivarium*. FIG. 5i shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 100) of *M. spermatophilum*. FIG. 5j shows results of hybridization reaction of genus-specific probe (SEQ ID No. 7) and species-specific probe (SEQ ID No. 122) of *U. urealyticum*. FIG. 5k shows results of hybridization reaction of genus-specific probe (SEQ ID No. 22) and species-specific probe (SEQ ID No. 128) of *A. laidlawii*.

### **Industrial Applicability**

As described above, the present invention provides a rapid and accurate assay method capable of simultaneously detecting many *Mycoplasma* and its related strains from a single sample using a microarray comprising novel oligonucleotides for detecting *Mycoplasma* and its related strains which are known as a source of contamination of cell lines and biological products and human pathogenic.

Also, the present invention provides an objective and credible assay method capable of tracing a contamination source for preventing expansion of infective *Mycoplasma* and its related strains and controlling a contamination of *Mycoplasma* against biological products and stem cells or cord blood cells which are useful for gene therapy and cell therapy.

Further, the present invention provides very specific and sensitive

hybridization assay for detecting *Mycoplasma* and its related strains using oligonucleotide probes designed based on sequence analysis of ITS region of many *Mycoplasma* Strains.

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